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Determination of Absorbed Dose of Ozone (03) in Animals and Humans Using Stable Isotope (Oxygen-18) Tracing

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16. ABSTRACT

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DETERMINATION OF ABSORBED DOSE OF OZONE (O_3) IN ANIMALS AND HUMANS USING STABLE ISOTOPE (OXYGEN-18) TRACING.

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Abstract

A method for the determination of absorbed dose of ozone (O_3) in animals and humans using oxygen-18 (^{18}O) as a physiological tracer is presented. The experimental aspects of the method are based on the instantaneous pyrolysis of tissue samples and subsequent conversion of the sample oxygen to carbon monoxide then to carbon dioxide whose isotopic composition is determined by isotope-ratio mass spectrometry. A mathematical procedure is then used to correct the isotopic data for interferences from the blank and memory effects and from the iodine pentoxide oxidation of CO to CO_2. Laboratory animals were exposed to $^{18}O_3$ (1 ppm, 1 hr) then tissues were dried and processed for ^{18}O measurement. Enrichments in ^{18}O over natural abundance ^{18}O was observed in lung homogenates, nasal cavities, tracnea, and pulmonary lavage fluids but not in blood of mice, rats, and rabbits. Thus, the ^{18}O tracing method appears to be sensitive enough to detect the reaction products of $^{18}O_3$ in animals exposed to near environmental concentrations of this gas.

INTRODUCTION

Ozone (O_3) is one of the most common photochemical oxidants and it also appears to be the most toxic. Its toxicity most likely arises from either direct reaction with tissue constituents or initiation of free radical-induced autoxidation. The toxicology data base on O_3 is extensive, particularly with regard to animal inhalation studies (1-4). There is a need to relate the animal toxicology data to human exposure conditions in order to provide more accurate risk assessments. Key information gaps in this area include the lack of sufficient data on differences between animals and humans in O_3 dose to target tissues, and a lack of information on molecular and cellular mechanisms of O_3 damage. Much of this uncertainty could be eliminated if it were possible to trace the fate of the O_3 molecule in the tissues as conveniently as in studies using radioactive isotopes. However, the lack of a long-lived radioactive isotope of oxygen which could be used as a physiological tracer has impeded tracing studies involving oxygen labels (Table 1).

Estimates for the dose of O_3 to specific sites along the respiratory tract following inhalation have been attempted based on mathematical models which account for both transport and absorption of oxidants in respiratory airways (5). These models agreed with experimental results in that the most likely sites of damage were the respiratory bronchioles and alveolar ducts (6), but quantitative verification of the predicted dose has not been possible. Previous studies funded by the EPA by

Santrock and Hayes made a significant contribution to this problem by tracing O_3 using the stable isotope, ^{18}O (7). Preliminary work on the implementation and improvement of this technique are presented here. Technique feasibility and accuracy were verified using isotopic standards prepared from benzoic acid. Different animal species (mice, rats, rabbits) were exposed in specially designed chambers in which ^{18}O enriched ozone was circulated. The efficacy of the method is suggested by preliminary animal data which show enrichment levels at different tissue sites that correspond to what expected based on the respiratory toxicity of O_3.

METHODS

Analytical Procedure

The method is based on the combination of the method of Unterzaucher (8) for the determination of oxygen in organic samples and isotope ratio mass spectrometry. Figure 1 shows a schematic diagram of the analysis steps. A commercial elemental analyzer (Carlo Erba 1106, H.B.I., Saddlebrook, NJ) was modified to allow the collection of analysis products to be directed to a glass vacuum circuit for cryogenic purification. After O_3 exposure, freeze-dried animal tissue is weighed in small silver cups and placed in the instrument automatic sampler. The ^{18}O enriched samples are then instantaneously pyrolyzed at 1060°C (9). The pyrolysis products pass through a specially packed carbon column

where quantitative conversion of all oxygen to carbon monoxide takes place. Because no oxygen is lost during this conversion, oxygen isotopic composition of the resultant carbon monoxide should be identical to that of the original organic sample. However, small interferences due to the memory effect (influence of previous samples on the present) and the blank effect (from oxygen released from the pyrolysis tube quartz walls) introduce errors to the isotopic abundance which can be mathematically corrected. After separation and quantitation of carbon monoxide in the gas chromatograph, it is then oxidized to carbon dioxide by passage through a column filled with iodine pentoxide at 120°C. The mixture of iodine pentoxide powder and glass beads is located after the gas chromatograph in the analysis circuit. The formed carbon dioxide is monitored by a thermal conductivity cell. The recording of the carbon dioxide peak starts the condensation step which occurs in the glass vacuum circuit. The circuit is maintained at high vacuum (10^{-7} Torr) by a pumping system consisting of a mechanical vacuum pump (Sargent-Welsh, Skokie, IL) and an oil diffusion vacuum pump (CVC, Rochester, NY). A dry ice trap is used to remove the I_2 produced in the carbon monoxide oxidation reaction. A series of cryogenic traps are used to condense the carbon dioxide into sealed glass tubes which are then analyzed by isotope ratio mass spectrometry (Finnigan San Jose, CA).

Mathematical Model for Interference Correction

Isotopic data are expressed as ^{18}O fractional abundance, F, which is defined as follows:

$$F = \frac{\text{number of oxygen-18 atoms}}{\text{total number of oxygen atoms}} = \frac{^{18}O}{^{16}O+^{17}O+^{18}O} \qquad (1)$$

A mathematical model relating the ^{18}O fractional abundance of the i-th sample, $F_{s,i}$, to that of the carbon dioxide is derived using balance equations describing the process:

$$F_{CO_2,i} = \frac{1}{2}\left[\frac{N_B F_B + \sum_{j=0}^{i-1} N_{s,i-j}\, e^{-jb}\, F_{s,i-j}}{N_B + \sum_{j=0}^{i-1} N_{s,i-j}\, e^{-jb}} + F_I\right] \qquad (2)$$

$$i=1,p$$

F_I and F_B are the fractional abundances of ^{18}O of the iodine pentoxide and the blank, respectively. The $N_{s,i}$ and N_B are the amounts of oxygen (μmoles O) in the ith sample and in the blank, respectively; p is the total number of samples.

This present mathematical model improves over the previous approach (7) in that it does not require memory equilibration conditions, it requires fewer assumptions and allows the simultaneous determination of the correction factors. Thus, it can be performed more frequently to assure that the entire analysis system is functioning in a reproducible manner.

The exponential term represents the clearance nature of the memory effect (10). Equation (2) represents a system of p non-linear equations with 2 independent variables, $N_{s,i}$ and $F_{s,i}$. The unknowns are the correction factors for the blank (N_B and F_B) and for the memory (b). These correction factors are determined using data from a set of samples of known isotopic composition which is then fitted to equation (2) using a non-linear least squares computer program. Once these parameters are obtained, their values are inserted into Equation (2) to allow the sequential determination of the organic sample fractional abundances. An expression for the $F_{s,i}$ is easily deduced by rearranging equation (2):

$$F_{s,i} = \frac{1}{N_{s,i}} \left[(2\,F_{CO_2,i} - F_I)\,(N_B + \sum_{j=0}^{i-1} N_{s,i-j}\,e^{-jb}) - N_B\,F_B - \right.$$

$$\left. - \sum_{j=0}^{i-1} N_{s,i-j}\,e^{-jb} \right] \qquad i=1,..p \qquad (3)$$

Benzoic Acid Calibration Standards

Validation of correction parameters for the conversion of tissue oxygen to CO_2 is accomplished through the use of benzoic acid isotopic standards that can be processed in the elemental analyzer similar to tissue samples or directly decarboxylated to CO_2 using a heating cycle. The benzoic acid standards were prepared by isotopic exchange with ^{18}O enriched water (11). Five different isotope abundance standards were prepared ranging from 0.2% to 0.40% ^{18}O. Samples of about 3 mg

were weighed to the nearest microgram using a microbalance. These samples were then introduced into acid-washed and pre-heated 6 mm O.D. pyrex tubes and heated in a muffle furnace at 550°C for 10 minutes. This heat treatment released the pyrolysis products: mainly benzene and CO_2 (13). A series of cryogenic traps was used to purify the carbon dioxide prior to isotope ratio mass spectrometry.

Ozone-18 Animal Exposure System

$^{18}O_3$ is produced from pure $^{18}O_2$ (Cambridge Isotopes, Aobern, MA) using an on-line procedure as depicted in Figure 2. A mixture of nitrogen and $^{18}O_2$ gases (95% N_2, 5% $^{18}O_2$) is introduced into a silent arc O_3 generator at a flow rate of 0.5 l/min. The formed $^{18}O_3$ is then diluted with purified air to yield a concentration of 1 ppm of $^{18}O_3$. An $^{18}O_3$ monitor (Bendix, Lewisburg, WV) is connected to the chamber to insure that this value remains constant during the entire exposure period (1 hour). This depicted exposure configuration presents an improvement over a previous technique that was based on cryogenic distillation of O_3 in a vacuum circuit (7) prior to transfer of the $^{18}O_3$ to the chamber using a syringe. The present method is both cheaper (no need to build a vacuum circuit for the purpose of generating ozone-18) and faster (the generated ozone is directed to the exposure chamber which is situated downstream). It is also safer since it eliminates the danger of ozone explosion when its condensed form is evaporated.

Tissue Preparation and ^{18}O Tracing Methods.

Immediately following inhalation exposures of the animals, tissues were removed, frozen in liquid N_2 and lyophilized. Lungs of rabbits were lavaged with a volume of saline equal to 35 ml/kg of body weight as described previously (12). The lavage fluid cells and surfactant pellets were obtained by centrifugation at 400g and 20,000g, respectively. Lyophilized heads of the animals were opened and the entire nasal epithelium and tissue surrounding the air passages was removed and lyophilized again. Dried tissues were further pulverized by shaking with small steel spheres placed in the tubes. Pulverized dry tissue was weighed into silver sample cups prior to the pyrolysis steps. Evaluation of data from tissues of $^{18}O_3$ exposed animals is most conveniently done by subtracting the ^{18}F of control tissues from the ^{18}F observed in the $^{18}O_3$ exposed tissue. This subtraction yields the "enrichment" in ^{18}O in the treated tissue which can be expressed in the units of μmoles ^{18}O per mole of total oxygen. From the enrichment measurements and the percentages of tissue oxygen, further calculations can be made regarding the number of moles of ozone-derived oxygen remaining in the tissues.

RESULTS

Direct pyrolysis of benzoic acid was found to yield isotopic results similar to pyrolysis of the same samples in the elemental analyzer. These results, which are shown in Table 2, confirm the efficacy of the

tissue pyrolysis methods. Standard samples were processed both by direct pyrolysis (column 3) or through the Carlo Erba elemental analyzer (Unterzaucher procedure, column 4). The latter data are corrected for memory, blank and I_2O_5 oxidation. The difference (error) between the two sets of data is listed in the 5th column. The close agreement between the two data sets supports the validity of the method. As expected, the error, Δ, increases with sample enrichment but remains small.

Spiking studies were also conducted to investigate the effect of impurities such as those found in biological samples on the accuracy of the method. Samples of isotopic standard BA-5, were mixed with a dry protein standard (gelatin) and the isotopic composition of the mixture compared with the composition of each individual component. Table 3 shows that the deviation from expected is small (1.76%). From all the standard assays, it was clear that the method should offer great promise for tissue samples.

Results of experiments shown in Table 4 demonstrate the reproducibility of the natural abundance ^{18}O measurements in lung tissue from mice and rats. Thus, natural abundance data from control animal lungs was used as the basis for expression of enrichments in ^{18}O. Mice, rats, and rabbits were exposed to $^{18}O_3$ for 1 hr and control animals were exposed to filtered room air for the same period. Data obtained to date suggest that no excess ^{18}O is measurable in the blood of mice and rats following

the exposure levels (Table 4). Table 5 shows the percentages of oxygen and the excess ^{18}O contents of selected respiratory tissues of $^{18}O_3$-exposed animals. Most of the tissues from the 3 animal species were measurably enriched in ^{18}O, including lung, trachea and excised nasal tissue homogenates. It appeared that tissues lining the respiratory tract were more highly enriched in ^{18}O than homogenized whole tissues. For example, rabbit alveolar macrophages and surfactant pellets were enriched more than the whole lung homogenates. Enrichments ranged from 5 to 50 µmoles ^{18}O/mole of total oxygen, which at the oxygen percentages of 15-20% is equivalent to excess ^{18}O levels of approximately 0.1 to 1.0 nanomoles per mg of dry tissue. Such small oxygen enrichments would not be detectable by oxygen elemental analysis alone, however, the combination of elemental analysis with isotope ratio mass spectrometry provided both sensitive and exact definition of the label derived from $^{18}O_3$.

DISCUSSION

Although isotopic data on ^{18}O enrichments in animals exposed to $^{18}O_3$ is sparse at present, it is evident that the procedures used have potential for tracing ozone reaction products *in vivo*. Excess ^{18}O appears to be easily detectable in the respiratory tract following $^{18}O_3$ exposures in the 1 ppm concentration range for 1 hr. Although excess ^{18}O has not been detectable to date in the blood of $^{18}O_3$ exposed animals, it is possible that the use of increased numbers of animals and improved isotopic correction parameters will enable detection of ^{18}O in blood.

Various mechanistic questions could become approachable using these labeled O_3 techniques. The uptake of ^{18}O into tissues of animals exposed to an atmosphere of $^{16}O_3$ combined with $^{18}O_2$ will be performed to determine the extent of spontaneous autoxidation that accompanies direct reaction of the O_3 molecule with the tissues. The relative concentrations of O_3 reaction products in DNA, lipid membrane components, and cytosolic enzymes will also be possible to determine using these techniques. This information should allow a more refined definition of the cellular targets for O_3 as well as improved sensitivity of dosimetric measurements. Until now, methods for measurement of oxygen "binding" into any or all fractions of a complex tissue have not been available.

Future studies will also include measurement of the uptake of ozone into the nasal and pulmonary lavage fluids in both animals and humans. Data from such studies will provide a direct link between animal toxicology data on O_3 and potential human toxicity. In conjunction with mathematical modeling procedures (5), these data should provide improved risk assessments in humans based on the animal toxicology data base.

In summary, the methodological refinements described here improve several aspects of oxygen 18 tracing procedures in complex tissues. These include improved exposure methods for labeled O_3, improved efficiency of sample preparation steps for tissue ^{18}O determination, and improved accuracy of isotopic measurements of ^{18}O in CO_2 derived from

the tissues. It is evident that tracing O_3 reaction products in vivo is feisible, yet it requires some unique experimental as well as mathematical approaches to provide meaningful data.

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TABLE 1

OXYGEN ISOTOPES

Nuclide	Natural Abundance %	Mass	Half Life (seconds)
^{13}O	-	13	0.0087
^{14}O	-	14	71.0
^{15}O	-	15	124
^{16}O	99.759	15.9949	-
^{17}O	0.037	16.999	-
^{18}O	0.204	17.999	-
^{19}O	-	19	29
^{20}O	-	20	14

TABLE 2

COMPARISON OF ISOTOPIC ANALYSES OF
BENZOIC ACID STANDARDS USING PYROLYTIC
DECARBOXYLATION AND THE MODIFIED UNTERZAUCHER PROCEDURE

Isotopic Standard	n	Pyrolytic Decarboxyl. $^{18}Fx10^3$	Unterzaucher Procedure $^{18}Fx10^3$	Δ (‰)
BA-1	10	2.0262 ± 0.0017	2.0227 ± 0.0054	-0.17
BA-2	10	2.1034 ± 0.0052	2.1114 ± 0.0079	+0.37
BA-3	5	2.4716 ± 0.0089	2.5032 ± 0.0188	+1.26
BA-4	5	2.9818 ± 0.0121	2.9480 ± 0.0207	-1.58
BA-5	7	3.9710 ± 0.0071	3.8476 ± 0.1081	-3.20

n = number of replicate samples analyzed

TABLE 3

GELATIN SPIKING EXPERIMENT

Sample	n	Measured Fract. Abund. $^{18}Fx10^3$	Expected Fract. Abund. $^{18}Fx10^3$	Deviation (%)
BA-5	5	2.9480 ± 0.0207	2.9818	-1.58
Gelatin	5	1.9986 ± 0.0021	-	-
BA-5/Gel.	5	2.4462 ± 0.0242	2.4902	-1.76

TABLE 4

ABUNDANCE OF OXYGEN-18 IN CONTROL ANIMAL LUNGS AND

IN BLOOD OF EXPOSED ANIMALS

Expt.	Animal, Tissue, Exposure	Fractional ^{18}O Abundance
1.	Mouse, lung, air	2.0013
		2.0006
		2.0020
2.	Mouse, lung, air	2.0021
		2.0006
	Mouse, blood, O_3	2.0020
		2.0016
		2.0016
		2.0019
3.	Rat, lung, air	2.0028
		2.0031
	Rat, blood, O_3	2.0030
		2.0024

TABLE 5

EXCESS OXYGEN-18 IN TISSUES OF ANIMALS EXPOSED TO 1.0 ppm $^{18}O_3$

Expt.	Animal	Tissue	n	Tissue Oxygen, % of dry wt.	^{18}O Enrichment μmoles ^{18}O/mole total oxygen
1	Mouse	Lung	5	19.3 ± 0.5	14.8 ± 7.8
2	Rat	Lung	5	18.6 ± 1.8	6.6 ± 6.4
		Trachea	4	21.3 ± 1.2	12.4 ± 8.9
		Nasopyarynx	4	19.5 ± 2.3	7.3 ± 1.5
3	Rabbit	Lung	2	19.9 ± 0.1	15.6 ± 3.5
		A. Macrophage	2	14.6 ± 0.4	45.2 ± 3.0
		Surfact. Pellet	2	10.1 ± 0.8	55.0 ± 6.5

Mean ± S.D. are shown for n animals. Exposure duration was 1 hr.

FIGURE LEGENDS

Figure 1. Schematic representation of the procedure for sample preparation and analysis for tracing ^{18}O in tissue samples. Tissue samples are pyrolyzed in a Carlo Erba elemental analyzer. After gas chromatographic separation, the carbon monoxide effluent of the analyzer is oxidized to carbon dioxide which is then cryogenically purified in a vacuum circuit. Isotope-ratio mass spectrometry of the resulting CO_2 and corrections to the data complete the analysis.

Figure 2. Schematic diagram of ^{18}O animal exposure system. $^{18}O_3$ is on-line generated in a silent arc ozone generator and mixed with purified air to yield a concentration of 1 ppm in the chamber. Due to the natural abundance of ^{18}O in air (0.2% of the oxygen is ^{18}O) normal room air contains 426 ppm of $^{18}O_2$. The incomplete conversion of $^{18}O_2$ to $^{18}O_3$ results in an approximate 13% excess amount of $^{18}O_2$ in addition to the 1.0 ppm $^{18}O_3$.

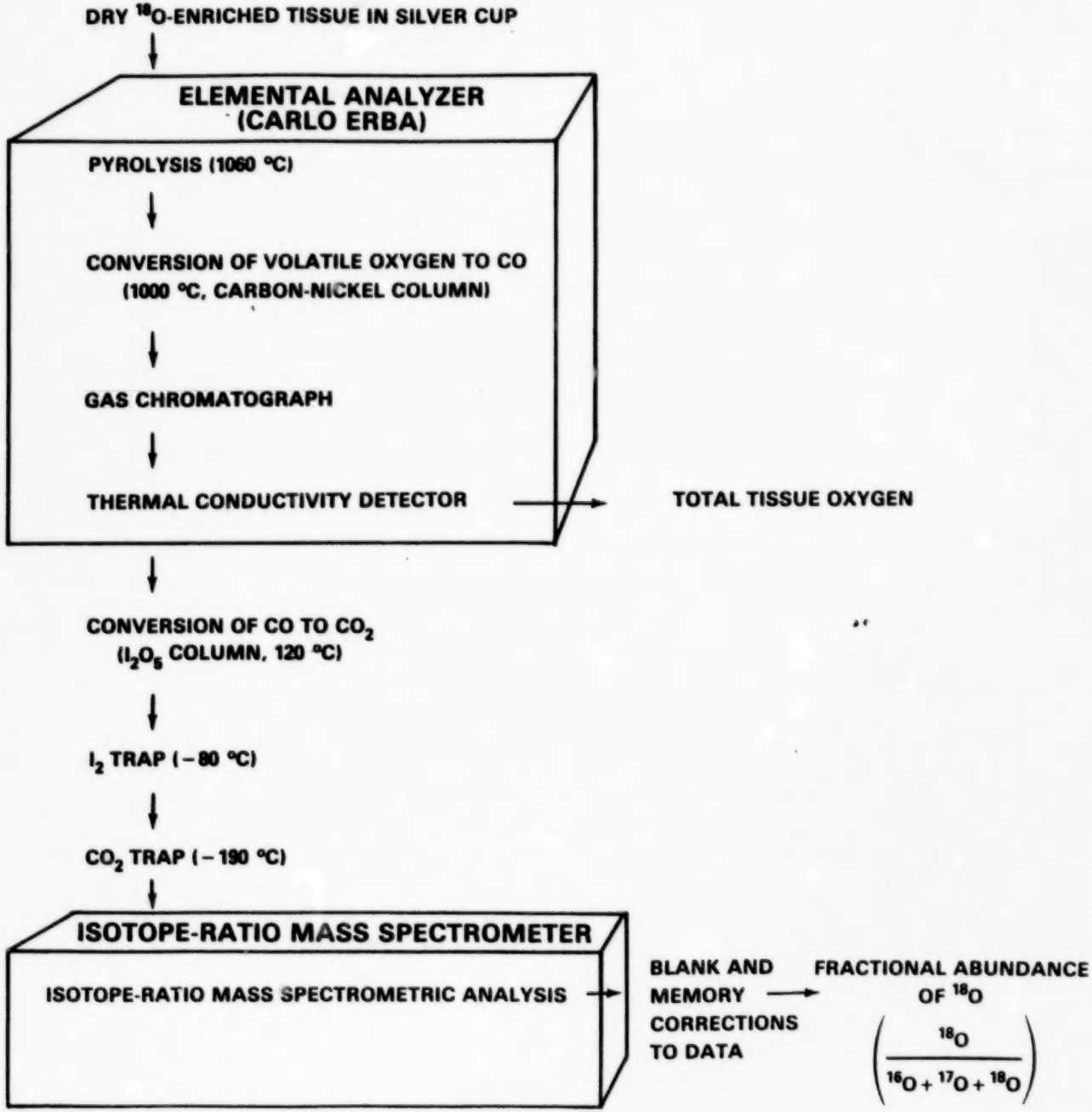
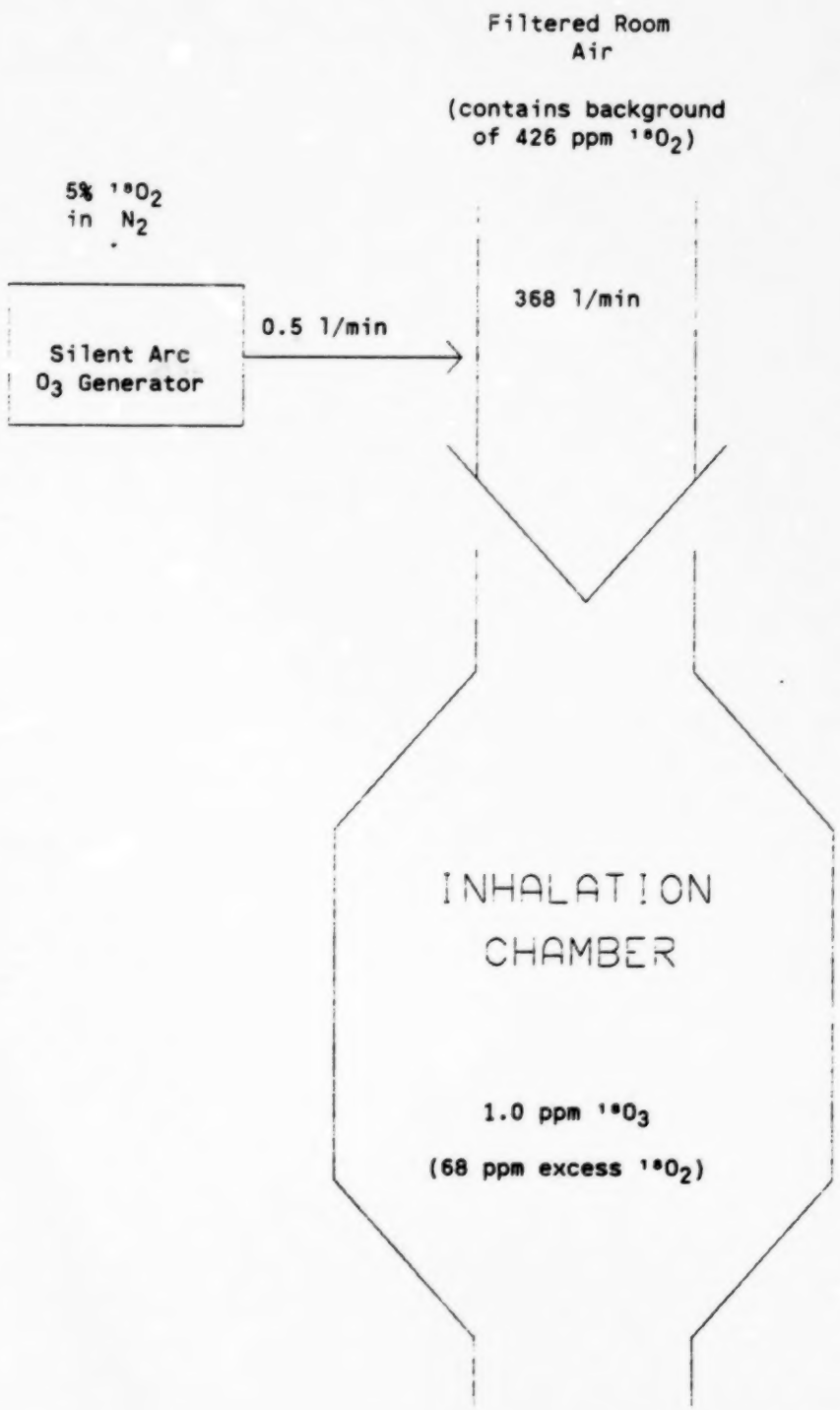

DRY ^{18}O-ENRICHED TISSUE IN SILVER CUP
ELEMENTAL ANALYZER (CARLO ERBA)
PYROLYSIS (1060 °C)
CONVERSION OF VOLATILE OXYGEN TO CO (1000 °C, CARBON-NICKEL COLUMN)
GAS CHROMATOGRAPH
THERMAL CONDUCTIVITY DETECTOR
TOTAL TISSUE OXYGEN
CONVERSION OF CO TO CO_2 (I_2O_5 COLUMN, 120 °C)
I_2 TRAP (−80 °C)
CO_2 TRAP (−190 °C)
ISOTOPE-RATIO MASS SPECTROMETER
ISOTOPE-RATIO MASS SPECTROMETRIC ANALYSIS
BLANK AND MEMORY CORRECTIONS TO DATA
FRACTIONAL ABUNDANCE OF ^{18}O
$\left(\frac{^{18}O}{^{16}O + ^{17}O + ^{18}O}\right)$

FIGURE 2


Filtered Room Air
(contains background of 426 ppm $^{18}O_2$)
5% $^{18}O_2$ in N_2
Silent Arc O_3 Generator
0.5 l/min
368 l/min
INHALATION CHAMBER
1.0 ppm $^{18}O_3$
(68 ppm excess $^{18}O_2$)